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Bacterial invasion of vascular cell types: vascular infectology and atherogenesis

Emil Kozarov
Section of Oral & Diagnostic Sciences, Columbia University Medical Center, 630 West 168 Street, P&S Box 20, New York, NY 10032, USA

Emil Kozarov: ekozarov@gmail.com

Abstract

To portray the chronic inflammation in atherosclerosis, leukocytic cell types involved in the immune response to invading pathogens are often the focus. However, atherogenesis is a complex pathological deterioration of the arterial walls, where vascular cell types are participants with regards to deterioration and disease. Since other recent reviews have detailed the role of both the innate and adaptive immune response in atherosclerosis, herein we will summarize the latest developments regarding the association of bacteria with vascular cell types: infections as a risk factor for atherosclerosis; bacterial invasion of vascular cell types; the atherogenic sequelae of bacterial presence such as endothelial activation and blood clotting; and the identification of the species that are able to colonize this niche. The evidence of a polybacterial infectious component of the atheromatous lesions opens the doors for exploration of the new field of vascular infectology and for the study of atherosclerosis microbiome.

Keywords

atherosclerosis; bacterial invasion; chronic inflammation; etiology; infection

Atherosclerosis as an inflammatory disease

Importance of the problem

Atherosclerosis (AS) is a chronic inflammatory condition associated with the presence of conventional cardiovascular risk factors such as hypercholesterolemia, hypertension, diabetes, smoking, and genetic factors. Cardiovascular disease (CVD, including AS and its sequelae) is an enormous burden to public health and to the US economy, costing US$448.5 billion in 2008 [201]. However, the incidence of AS is not fully explained by these risk factors [1]. It is now becoming clear that, in addition to the smooth muscle cells (SMCs) that play a major role in the progression of atherosclerotic lesions, leukocytes, growth factors and inflammatory mediators participate in a dynamic and progressive process, beginning with endothelial dysfunction and characterized by inflammation, as a key regulatory process that also interacts with the standard risk factors, leading to clinical symptoms of disease [2,3].

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The recognition of the inflammatory characteristics of AS led to the successful application of C-reactive protein (CRP) as an acute-phase marker for cardiovascular risk assessment [4]. Further introduction of diagnostics and treatments targeting vascular inflammation is dependent on advances in investigations relating to the origins of inflammation. Specifically, the contribution of bacterial pathogens to vascular inflammations, based on the latest communications as described herein, is attracting increasing attention.

Vascular infectology: are infections a risk factor for AS?

The inflammatory character of the atherosclerotic lesion has turned attention towards infection as a potential cause. The notion that AS is related to infections dates back to the 19th century, based on the results of an animal model, where infection of a rabbit resulted in the formation of fatty streaks [5]. A century later, the interest was rekindled again by animal experiments [6] and other investigations and prompting this idea to reemerge [7]. Since then, in addition to the recognized risk factors listed above, numerous studies have indicated infections as one of the contributing factors resulting in acute ischemias [8,9].

Indeed, an abundance of epidemiological evidence has been presented in support of this idea [9–11]. Serological evidence of association of bacteria with chronic coronary heart disease (CHD) and myocardial infarction (MI) was presented for the first time in *The Lancet* [12]. Furthermore, the results of a longitudinal seroepidemiological study of 572 patients where the extent of AS was measured by coronary angiography, carotid duplex sonography and anklearm index, consistently suggested that elevated IgA and IgG titers to infectious agents are associated with the extent of AS and with CVD death. After adjustment was made for age, sex, classical risk factors and high sensitivity CRP, infectious burden was significantly associated with advanced AS, with an odds ratio (95% CI) of 1.8 (1.2–2.6) for four to five seropositives ($p < 0.01$) and 2.5 (1.2–5.1) for six to eight seropositives ($p < 0.02$). Finally, after a mean follow-up of 3.2 years, CVD mortality rate in patients seropositive for up to three pathogens was 7.0% versus 20.0% in patients seropositive for six to eight pathogens [13]. IgM antibodies specific for phosphorylcholine, hapten-like epitope which is exposed on oxidized LDL and also on bacteria are atheroprotective and their low titers are associated to an increased risk for CVD [14]. Recently, the evidence that the aggregate burden of chronic infections, exacerbated by the host's immune response and may contribute to AS, led to the recognition of what is now known as 'pathogen burden' [15].

Oral diseases & systemic inflammations

Epidemiology

Observational studies have shown a particular association of systemic inflammation and endothelial dysfunction with periodontal inflammations [16,17]. To address the confusion due to similarities between periodontitis and CVD risk factors such as advanced age, diabetes, being male, smoking and low socioeconomic status, specific studies have been designed [18,19].

Indeed, the results from the INVEST study indicate that chronic infections, including periodontitis, may predispose individuals to CVD [20]. In a study of 657 subjects with no history of stroke or MI, mean carotid artery intima-media thickness was related to total bacteria burden, periodontal bacterial burden and the relative predominance of periodontal/over other bacteria in the subgingival plaque. Adjustment was made for age, race/ethnicity, gender, education, body mass index, smoking, diabetes, systolic blood pressure and LDL and HDL cholesterol. The results demonstrated that periodontal bacterial burden was related to the carotid intima-media thickness, a measure of subclinical AS ($p = 0.002$) and was further corroborated by Renvert *et al.* who demonstrated that total oral bacterial load was

higher in coronary disease subjects (mean difference: 17.4×10^5; standard deviation: 10.8; 95% CI: 4.2 to 17.4; $p < 0.001$), and significant for 26 species including *Porphyromonas gingivalis*, *Tannerella forsythensis* and *Treponema denticola* [21]. Furthermore, using a fully adjusted multivariate logistic regression model, periodontal bone loss was associated with an approximate fourfold increase in risk for carotid AS (adjusted odds ratio, 3.64; CI: 1.37–9.65) [22] and coronary disease subjects in a study of 90 males had significantly deeper pockets (2.28 vs 2.96 mm; $p < 0.001$) and greater attachment loss (2.85 vs 3.65 mm; $p < 0.001$) [23]. Edentulousness was independently associated with the risk of aortic stenosis in a cohort of 2341 individuals [24]. The majority of the epidemiological studies linked periodontal disease (PD) to increased incidence of cardiovascular, cerebrovascular and peripheral vascular disease [19].

Seroepidemiology

In addition to epidemiological data, serological evidence of association of bacteria with CVD keeps growing [25]. Interestingly, judging from strain-specific antibody titers, only the presence of specific *P. gingivalis* strains may promote atherogenesis [26]. As a recent example, infection by *Aggregatibacter actinomycetemcomitans* or *Chlamydophila pneumoniae* or host response against them have been associated with coronary obstruction [27] and elevated levels of *P. gingivalis* and *A. actinomycetemcomitans* – specific serum IgG were associated with AS [28]. Specifically, the resident dendritic cell interaction with phosphorylcholine- and cardiolipin-specific IgG2 bound to microorganisms and with microbial products may enhance dendritic cell IL12 production, IFN-γ response, macrophage (Mϕ) activation, chronic inflammation and eventually, plaque destabilization [29].

Meta-analyses

The epidemiological data were supported by meta-analyses; one such examination of PubMed, Cochrane Controlled Trials Register®, EMBASE®, and SCOPUS® databases for references on PD and CVD showed strong association of PD with CHD, with a summary odds ratio of 1.75 (95% CI: 1.32–2.34; $p < 0.001$), compared with subjects without PD [30]. Interestingly, other studies found stronger association of PD with stroke than with CHD (hazard ratio: 3.52; 95% CI: 1.59–7.81) [31].

Systemic effects of focal inflammations

There are two main metastatic pathways for systemic effects of focal inflammations, systemic bacteremia (direct effect) and spreading of inflammatory mediators, released in response to the infection at the diseased site (indirect effect, or immunological sounding). The indirect effects of the mediators have been subject to recent reviews [32,33]; here we will focus on the evidence of direct effects of bacteremias on vascular tissue.

Periodontal bacteremias

The initial event in bacteremia is the entry of bacteria into the circulation through the diseased site. The most studied model is transient bacteremia with oral pathogens entering through the microvasculature following tooth brushing and other dental procedures [34]. For example, blood samples taken from 30 patients before and after a procedure demonstrated bacteremia following ultrasonic scaling, periodontal probing and tooth brushing of 23, 16 and 13%, respectively, using PCR [35]. Extending the notion, a study of 194 patients showed that the periodontal site bleeding after tooth brushing was associated with an approximate eight-fold increase in bacteremia and that patients with mean plaque and calculus scores of ≥2 were at a 3.78- and 4.43-fold increased risk of bacteremia, respectively [36]. Collectively, the data suggest the possibility of repeated daily encounter between

invasive oral pathogens and endothelia, in particular in the areas of turbulent flow, which are exactly where atheromatous lesions form.

Endothelial response to injury

Atherosclerotic development, since it involves both the innate and adaptive arms of the immune system, bears similarity to bacterial infection. The 'response to injury' hypothesis of atherogenesis describes an inflammatory process leading to endothelial activation, growth factor release, monocyte adhesion and migration while maturing into macrophages, followed by foam cell formation and SMC proliferation. The inflammation is not only thought to cause initiation and progression of the atheroma, but the thrombosis and acute ischemic events leading to death are also inflammation-related (Figure 1) [37].

In concordance, high sensitivity CRP (a marker for inflammation) has been recognized as an important prognostic factor for cardiovascular disorders, such as atrial fibrillation [38] and AS [3]. However, in contrast to the mounting evidence that AS is a chronic disease induced and exacerbated by infectious agents, there is a paucity of experimental data regarding the actual association of pathogens with vascular inflammation. Evidence of bacterial presence at the diseased site (Koch's postulate) is required to determine a causal relationship and develop appropriate diagnostics and treatments.

Notably, there have been attempts to isolate and identify bacterial pathogens from atheromatous tissues. As a result, *C. pneumoniae* has been successfully cultivated from atheromas [39]. In light of this finding, antibiotic treatment was promptly proposed to achieve a significant improvement in disease outcome.

Antibiotic clinical trials

Intriguingly the results of the large randomized placebo-controlled clinical trials did not support the infectious agent hypothesis, consequently it was largely concluded that *C. pneumoniae* was not an important cofactor in AS and that efforts to treat *C. pneumoniae* with antimicrobials could not alter disease outcomes [40–42]. However, the following observations could not be ignored. Firstly, the accumulating epidemiological, seroepidemiological and animal model data support the hypothesis that infections contribute to AS [43]. For example, the NOMAS, a prospective cohort study of stroke incidence and prognosis, indicated that 'infectious burden' is associated with risk of stroke [44] and carotid plaque thickness [45]. Second, AS has the characteristics of a chronic inflammatory disease. Clinical studies have indicated that infections with multiple pathogens result in chronic persistent inflammation [9]. Third, internalization of many types of bacteria can produce a 'privileged niche', where they persist in a latent, nonreplicating state, sheltered from humoral and cellular immune surveillance [46], the most studied case being *Mycobacterium tuberculosis* [47]. Fourth, the antibiotics that were used may not have been active against all the pathogens at the site, especially when DNA data suggest that various pathogens are associated with atherosclerotic tissue [48,49]. Thus, the eradication of one species may not remove the source of the inflammation. Indeed, increase of cardiovascular mortality rate according to the number of infections to which a patient has been exposed was observed [13]. Lastly, as we will present evidence herein, the treatment may have been ineffective since the bacteria can be in a dormant antibiotic-tolerant state, such as the one described in biofilms [50]. Therefore, solving the problem of bacterial cultivation would both identify potential causative agents of inflammation and allow consideration of antimicrobial strategies in AS treatment.

Bacterial invasion of vascular tissue

Infectious agents are implicated in the etiology of various vascular conditions via multiple mechanisms including direct microbial invasion of endothelial cells, host cell activation and stimulation of leukocyte influx and of crossreactive B and T cells [51]. Bacterial invasion of vascular tissue leading to intracellular localization bears multiple advantages for the pathogen. Tissue invasion is very likely a key virulence property for a bacterium since it provides: a 'privileged niche' with access to host protein (nutritional) and iron substrates; a sequestration from the humoral and cellular immune response; and a means for persistence that is essential for a chronic pathogen.

Direct versus macrophage-mediated bacterial dissemination

In direct bacteremic dissemination, bacteria spread in the circulation and gain access to the endothelia. Conversely, bacteria internalized in monocytes/macrophages or in dendritic cells at the diseased site can utilize a 'Trojan horse' approach, disseminating to endothelia, where they can find a way to the lamina and tunica media due to extravasation of the carrier phagocytes. Fittingly, it has recently been proposed that *P. gingivalis* subverts normal dendritic cell function, promoting a highly migratory immunosuppressive dendritic cell phenotype that contributes to the metastatic spread from the oral sites and to inflammation in AS [52]. Similarly, the infection with invasive bacteria has been shown to induce monocyte migration and significantly enhance the production of proinflammatory cytokines [53].

Bacterial invasion of vascular cell types

Certain bacteria have evolved to invade nonprofessional phagocytic cells. The process of bacterial invasion of host tissue begins with adherence to the target cell. Many pathogenic bacteria have acquired a large and diverse set of adhesive moieties on their surfaces in order to recognize and bind to specific host cell surface receptors [54]. Attachment is a prerequisite for entry, therefore it is part of the same continuous process. For specific information on adherence/attachment, there are multiple reviews detailing the variety of molecular intermediaries and mechanisms of bacterial adhesion to host cells [55–57].

In association with AS, most studies of anaerobic and facultative intracellular pathogens have utilized the main 'suspects', periodontal bacteria. Previously, *P. gingivalis*, *Eikenella corrodens* and *Prevotella intermedia* that are known members of the periodontal microbiota, were shown to invade bovine and human primary endothelial and SMCs [58,59]; the former group demonstrated for the first time that fimbriae were important for the process and the latter demonstrated for the first time, using immunofluorescence microscopy, that *P. gingivalis* was routed intracellularly to the autophagic pathway (see Box 1) [60]. This organism also triggers apoptosis in the host's endothelium [61]. Importantly for chronic inflammation such as AS, we have demonstrated the extended intracellular survival of *P. gingivalis* in vascular cells [62].

In addition to periodontal species, other oral bacteria detected in bacteremias, including several streptococcal species, have also been shown to invade coronary artery endothelial cells, for example *Streptococcus mutans* [63]. In concordance, we and others have also identified *S. mutans* DNA in atheromatous tissues [48,64].

Lipid raft-mediated internalization can be followed by usurping the autophagic pathway. Using receptor inhibitors, recombinant proteins and fluorescence microscopy, the meningitic *Escherichia coli* invasion of brain endothelial cells was shown to be lipid raft-dependent (see Box 2) [65].

Once internalized, bacteria may be targeted toward the autophagic pathway, however many intracellular pathogens seem to exploit or subvert this pathway – *P. gingivalis* [60,66], *Listeria monocytogenes* [67], *M. tuberculosis* [68] and *Coxiella burnetii* [69]. In human coronary artery endothelial cells, internalized *P. gingivalis* localizes within autophagosomes and prevents the formation of autolysosomes. These findings suggest that stimulation of autophagy and vitamin D3-mediated innate immunity may confer protection against intracellular pathogens. Since autophagy has been shown to be essential for elimination of intracellular bacteria and viruses [70], it may be exploited to treat chronic infections.

Bacterial transmission between vascular cell types

In a thorough investigation of *P. gingivalis* invasion of primary human endothelial and SMCs, we have shown that the organism can spread intercellularly, similarly to infection and spreading in gingival tissue [71]. The bacteria can transmit between the same, as well as between different cell types. Cell–cell contact between infected cells and new host cells increases the rate of transmission, although bacteria can cross significant distance in medium as well [62].

Proatherogenic sequelae of bacterial presence in vascular cells

Endothelial & SMC activation

The presence in the vascular wall of bacterial pathogen-associated molecular patterns stimulates the toll-like receptors (TLRs) of the leukocytes leading to release of proinflammatory cytokines contributing to atherogenesis [72]. The induction of proinflammatory mediators is species-specific, since *Tannerella forsythia* and *Treponema denticola* induced a much lower level of MCP-1 in endothelial cells than infection with *P. gingivalis* [73]. *P. gingivalis* infection activates host cells using TLR2 and TLR4 for cell signaling [32,74]. Vascular cell activation is a pivotal moment in initiation of atherogenesis. Using FACS and confocal microscopy, it was shown that the expression of ICAM-1, VCAM-1 and P- and E-selectins was induced in human endothelial cells infected with *P. gingivalis*, but not with noninvasive nonfimbriated mutant [75]. Importantly, it was shown by the same group that fimbria elicit chemokine production in human aortic endothelial cells (HAEC) via actin cytoskeletal rearrangements and that the proinflammatory IL1β, IL8 and MCP-1 were induced [76].

Further upgrading this investigation to the whole genome level, using high-density oligonucleotide microarrays, the transcriptome profile of HAEC after infection with invasive *P. gingivalis* strain 381 and noninvasive FimA mutant was defined. Invasive *P. gingivalis* upregulated 68 genes, compared with the mutant with four genes. The genes coding for Gro2 and Gro3 cytokines, for ICAM-1, for VCAM-1, for ELAM-1 (E-selectin), for IL8 and for the proinflammatory IL6 and COX2 were upregulated compared with uninfected HAEC control. Using PCR, immune assays and FACS, the expression of ICAM-1, VCAM-1, E-/P-selectins, IL6 and IL8 in HAEC infected with invasive *P. gingivalis* was confirmed, demonstrating that *P. gingivalis* fimbria-mediated invasion upregulates inflammatory gene expression in HAEC and accelerates inflammatory responses in the aorta [77]. Triggering of apoptosis in infected endothelial cells is another mechanism of tissue damage in vasculopathies. *P. gingivalis* infection induced apoptosis in human endothelial cells in a dose-dependent manner [61]. In addition, it has been shown that the vast majority of dying cells in advanced atherosclerotic plaques undergo necrosis, contributing to plaque instability by releasing tissue factor and matrix metalloproteinases [78]. We have shown endothelial cell layer's disruption, cell death and sharp drop in transendothelial resistance in polarized cell layers treated with *P. gingivalis* secreted protein fraction (gingipains) [79].

The SMCs respond to bacteria in a prothrombotic manner [80] (see below) or in a proliferative manner [81]. Interestingly, in the latter case, SMC proliferation in distal aortic aneurysms was associated with the presence of *P. gingivalis* in the dental plaque of the patients.

Prothrombotic effects of bacteria

An alternative plausible mechanism through which bacteremias (or bacteria present in ruptured plaque) may contribute to vascular thrombosis is the triggering of the coagulation cascade, as described in other articles [34,82]. Peripheral artery disease, also caused by endothelial dysfunction and inflammation, is not only one of the most common manifestations of AS, but is also a powerful and independent predictor of cardiac and cerebral ischemic events. It is caused by a fibrous clot occluding the femoral arteries putting the affected patients at high risk of mortality [83].

The potential adverse role of *P. gingivalis* in atherothrombosis has been demonstrated using human aortic SMCs. Live invasive, but not heat-killed or noninvasive mutant bacteria specifically suppressed tissue factor pathway inhibitors produced by vascular cells, suggesting a procoagulant response of the host cells to bacterial challenge [80].

Plaque rupture

The contribution of bacteria to the most critical moment in atherogenesis, plaque destabilization and rupture, is not limited to the inflammatory process alone. Plaque rupture, leading to leakage of the prothrombotic plaque core in the circulation and thrombus formation, can be due to the bacteria-dependent release of matrix metalloproteinases with concomitant suppression of the matrix metalloproteinase antagonist, tissue inhibitor of metalloproteinases [84,85].

Animal models can serve as a link between hypotheses and clinical practice [86]. Importantly, although both wild-type *P. gingivalis* and the noninvasive FimA-mutant were detected in blood and aortic tissue of $ApoE^{-/-}$ mice by PCR after challenge, it was reported that only the invasive strain accelerated AS in a murine model [87]. Importantly, the same study demonstrated prevention of *P. gingivalis*-accelerated AS via immunization to control *P. gingivalis*-elicited periodontitis.

Furthermore, in a recent investigation using a mouse model of AS and drug administration followed by *P. gingivalis* intravenous inoculation, two important hypotheses were tested using $ApoE^{(+/-)}$ mice [88]. First, it was shown that the observed absence of aortic lesions in the animals inoculated with fimbriae-deficient strain (DPG3) compared with wild-type strain (381) and the reduction in serum cytokine levels of IL1α, IL1β, IL6, IL18, TNF-α, VEGF and others may be due to lack of invasion ability of the fimbrial protein FimA-deficient strain DPG3. Second, it was proven that metronidazole, common antibacterial used in anaerobic infections, including periodontal, completely prevented the formation of *P. gingivalis*-associated aortic lesions. This indicates that invasive bacteria, and in particular this oral pathogen is capable of exerting a critical damage on the vessels and that drugs can be considered among the treatment options. This elegant study has implications beyond the direct pharmacotherapeutic applications. It suggests that the bacterial contact with endothelia and the subsequent invasion and activation of vascular endothelium that may lead to leukocyte recruitment to the infection site is a key step in the initiation and progression of atherogenesis.

Association of bacteria with atherosclerotic lesions

Bacterial DNA fingerprints in atheromatous tissue

Identification of the species that may be able to colonize this niche has been carried out using PCR and metagenomic approaches.

As discussed above, bacteria can enter the circulation through the microvasculature following tooth brushing and other dental procedures [36]. More than 700 bacterial species inhabit the mouth [89,90]. Of note, periodontal tissue bleeding seems most associated with bacteremia, while the measures of periodontitis are not [36]. Similarly to the previous observations that bleeding as a result of probing is more associated with systemic inflammation than attachment loss [91]. There is strong evidence that some species, such as *P. gingivalis* are disseminated to large vessels, since *P. gingivalis* DNA can be detected in atheromas [92]. Using PCR, we have found that 1.5–2.2% of the total DNA in the atheromatous samples was bacterial, and that a large proportion of it was of oral origin, especially in the elderly group of individuals, with *P. gingivalis* being the most represented [48]. It was expected, since severe PDs (≥4 mm attachment loss) increase in prevalence with age, with approximately 50% of 55–64 years olds having evidence of severe disease. Coincidentally, this is the age group with the highest incidence of MI and stroke.

Metagenomic screening of the atheromatous samples

As an example of the metagenomics approach, we are currently analyzing the phylogenetic diversity of bacteria present in atheromatous samples using 16S rDNA sequencing. The totxal genomic DNA from the tissues and the matching controls were isolated using a QIAamp® Tissue Kit (Qiagen, Valencia, CA, USA) as described [48]. A full-length segment of 16S rDNA was amplified from the total DNA samples using Advantage® 2 Polymerase mix (Clontech, Mountain View, CA, USA) with universal 16S rDNA primers [93]. The resulting 1.5 kb PCR fragments were isolated after electrophoresis in agarose gels, subcloned using TOPO® TA Cloning Kit (Invitrogene, Carlsbad, CA, USA) and sequenced. The complete sequences of the 16S rDNA segments were assembled and analyzed using Ribosomal Database project 16S RNA classifier [94] at the Ribosomal Database project website (Figure 2) [202] [Kalachikov S, Rafferty B, Kozarov E (2012), Manuscript in preparation].

In a metagenomics study of 16S rDNA signatures in atherosclerotic tissue from 38 patients with CHD, bacterial DNA from more than 50 species was present in all CHD patients but not in control material or in any of the normal/unaffected coronary arteries. The bacterial presence in atherosclerotic lesions was also confirmed by FISH [49]. In another study, using PCR, periodontal bacteria from 12 different patients were compared with the bacteria detected in the atheromatous plaques from the same patients. In two patients, *A. actinomycetemcomitans* was found both in the periodontal and in the atheromatous plaques [95]. Finally, in the recent most comprehensive metagenomics investigation to date, 454 pyrosequencing of 16S rRNA genes from atherosclerotic plaques and oral and gut samples of 15 patients with AS were undertaken to assess the bacterial diversity in the atherosclerotic tissue, in addition to oral and gut samples of healthy controls. The combined abundances of *Veillonella* and *Streptococcus* in atherosclerotic plaques correlated with their abundance in the oral cavity and several additional bacterial phylotypes were common to the atheromatous tissue and the oral or gut microbiota of the same individual [96].

Immunological detection of bacterial pathogens in atherosclerotic plaques

Using monoclonal antibodies, the presence of six bacteria, including periodontal pathogens, was detected in plaque sections [97], thus supporting the DNA data at the protein level.

However, the presence of species-specific antigens needs to be complemented with demonstration of viability of the organisms in order to build causative associations with the disease.

Cultivation & identification of bacterial strains from atheromatous tissues

The presence of bacterial 16S rDNA fingerprints in atheromatous tissues may simply be due to tissue macrophages carrying their refuse, phagocytized bacteria, from another infection site, therefore cannot argue for causality. In addition, DNA does not mean viability. To fulfill Koch's postulate, cultivation of bacteria from diseased tissue must be demonstrated to provide mechanistic data linking infectious organisms to CVD. Such cultivation has been eluding the biomedical community for decades and as a result (with the exception of *C. pneumoniae*) clinical strains could not be cultivated to provide key mechanistic link suggesting causality [98].

Since we observed viable invasive bacteria in atheromas [99] and uncultivable but viable intracellular bacteria internalized in vascular cell types (endothelial and SMCs) [62], we hypothesized that bacteria may become uncultivable when they reside intracellularly in atheromatous tissues. Indeed, leukocytic cells, which reside in these tissues, can harbor intracellular *M. tuberculosis* [100]. Moreover, uncultivable *Legionella* sp. have been made cultivable by coincubation with amoeba [101] and *Tropheryma whippelii* has been cultivated by coincubation with human monocytes [102]. Thus, we hypothesized that the human monocytic cell line THP-1 can facilitate recovery of uncultivable bacterial species from human atherectomy tissues, and processed such tissues accordingly.

Altogether we isolated a total of 872 bacterial isolates from seven atheromatous specimens [103]. Specimens cocultivated with monocytes versus control tissue yielded 124 versus 22 colony-forming units on average with the median 140 versus seven, respectively ($p < 0.05$). Monocyte cocultivation with plaque homogenate from patient one followed by lysis and anaerobic incubation produced 20 *P. gingivalis* colonies in addition to 236 pale colonies with several different morphological types, determined to be *Propionibacterium acnes*, *Staphylococcus epidermidis* and *S. infantis*. Treatment of additional tissue from patient one yielded a total of 106 *P. gingivalis* isolates. No black-pigmenting colonies grew in nontreated controls. In addition, we tested total AS tissue DNA from patient one for *P. gingivalis* DNA using quantitative PCR of two genes (16S rDNA and *rag*A) as described [48] and found that 1 g of tissue contained $\geq 3.5 \times 10^3$ *P. gingivalis* [103].

Interestingly, *P. acnes*, also a member of the periodontal microbiota is the most prevalent species in apical periodontitis lesions [104] and has been recovered from root canals and from blood samples taken during and after endodontic treatment [105]. Using multivariable regression models, it has been shown that among patients with 25 or more teeth, those reporting having had endodontic therapy two or more times had 1.62 (95% CI: 1.04–2.53) times the odds of prevalent coronary disease compared with those reporting never having had endodontic therapy [106]. It is also not surprising to recover *Staphylococcus* or *Streptococcus*, commonly observed in other systemic conditions; the staphylococci in prosthetic valve endocarditis [107] and the streptococci in heart valves and atheromas [48,64]. In addition, in a case of a septic patient, the only organism isolated from the atheromatous specimen was *Enterobacter* [108].

Can AS be a bacterial infection?

In contrast with the results from PCR or meta-genomics-based detection methods, yielding a large number of DNA signatures, we discovered that only a small number of bacterial species could actually be cultivated [103]. This could indicate that either not all viable

species were isolated or more importantly, that the inflammation may be associated with a limited spectrum of organisms only. The former is a remote option since: to have all 50+ species (whose DNA has been reported [49]) inhabiting the vascular tissue of plaque carriers (the majority of population) would severely compromise its sterility; and even in the case of a septic patient, we were only able to recover one GI tract species from atheromatous tissue [108]. Therefore, an exciting benefit of our approach could be a sharp reduction in false positive findings resulting from 16S rDNA signature analyses and limiting the possible contributing agents of disease to a small number of species. Our results will allow designing qualitative and quantitative studies of the association of specific bacteria with (clinical measures of) the disease [103].

Based on the recently communicated data, a particular model of atherogenesis emerges where bacteria invade endothelia either directly, as a result of bacteremia, or are carried by phagocytes (the 'Trojan horse' approach) (Figure 3). Upon invasion of endothelium, a limited number of bacterial pathogens (such as *P. gingivalis*) are able to reside inside the host cells for an extended period of time, activating the host tissue and initiating the atherogenic process. Within 24–72 h the bacteria switch into a dormant uncultivable stage (latency; see Box 3), in order to sustain their numbers and to persist. However, when facing a hostile environment (phagolysosomal fusion), some bacteria escape from the dormant stage, egress/exit into extracellular millieu and invade adjacent host cells, becoming transiently invasive and cultivable, and perpetuating persistent low-grade inflammation. The cell–cell transmission takes place even when the new cells were away from the infected cells. The return to cultivable state specifically occurs after engulfment by phagocytes, which additionally ensures metastatic dissemination, injurious response, apoptosis and necrosis, thus perpetuating the chronicity of disease.

The novelty of the infection-exacerbated model of atherogenesis is in the evidence for the presence of a variety of pathogens in atheromatous tissues, and in the proposed actual mechanisms of bacterial persistence in such tissues. Using such mechanisms, the anaerobic and facultative intracellular pathogens we found in atheromas would control their population in vascular tissues yet allow for the observed persistent infection. Importantly, dormant bacteria have low metabolic activity, thus, resembling the properties of biofilm bacteria they lack a target for antibiotics and therefore are drug-tolerant.

Clinical studies

The attractive premise of periodontitis being a modifiable risk factor focused multiple case-controlled, cohort and cross-sectional investigations on the systemic effects of periodontal inflammations. In retrospect, confirming that the infections can be a component of AS, intensive periodontal treatment reduced blood pressure and systemic inflammatory markers and improved lipid profiles with subsequent changes in cardiovascular risk [109]. The same group reported that such treatment of 61 patients, compared with 59 with community-based periodontal care, initially resulting in short-term systemic inflammation and endothelial dysfunction, leads to both oral health benefits and to improvement in endothelial function 6 months post-therapy (difference, 2.0%; 95% CI: 1.2–2.8; $p < 0.001$). The degree of improvement of endothelial function was associated with improvement in measures of PD ($r = 0.29$ by Spearman rank correlation, $p = 0.003$) [110]. Similarly, in a study of 61 individuals, successful periodontal treatment resulted in a significant improvement in flow-mediated dilation of the brachial artery (9.8% ± 5.7%; $p = 0.003$ compared with baseline) accompanied by a significant decrease in CRP concentrations (1.1 ± 1.9 vs 0.8 ± 0.8 at baseline, $p = 0.026$) [111]. Most importantly, periodontal therapy led to peripheral blood monocytes gene expression in a manner consistent with a systemic anti-inflammatory effect [112] and to reduction of $CD14^+$ blood monocytes by 47% ($p < 0.05$) and of the percentage of macrophages releasing TNF-α by 78% ($p < 0.05$). The serum inflammatory markers high

sensitivity CRP and soluble E-selectin also decreased by 37% ($p < 0.01$) and by 16.6% ($p < 0.05$) [113]. Periodontal therapy also improved the systemic inflammation and endothelial dysfunction in systemically healthy subjects [16] and resulted in a significant improvement of inflammation biomarkers levels, and of adhesion and activation proteins, while the intima-media thickness was significantly diminished [114].

Our model is also supported by the results from a double-blind, placebo-controlled study of 141 patients with acute infarction or unstable angina pectoris, treated with clarithromycin for 3 months [115]. The long-term clarithromycin treatment reduced recurrent cardiovascular events in periodontally healthy subjects, but failed to show any effect in subjects with periodontitis. It suggests that as a chronic polymicrobial infection, periodontitis may overpower the beneficial effects of antibiotics and that periodontally healthy CVD patients could still benefit from such treatment [116]. Although the discussed studies recorded indirect clinical outcomes or surrogate measures of AS and not primary outcomes (e.g., MI or stroke), overall, the value of periodontal interventions in the prevention of systemic diseases seems positive [117].

Conclusion

This brief presentation of the crucial field of infections in AS intends to lay out the latest on the infectious origins of this chronic inflammatory condition. Since, in addition to the accumulated so far evidence, we have detected viable bacteria and bacterial DNA in atheromatous vascular tissue, shown bacterial transmission between primary vascular cell types and for the first time cultivated variety of species from atheromatous tissues, here we argue that atherosclerotic initiation and development may be at least partially due, or is exacerbated, by infectious agents, which therefore represent an emerging ubiquitous risk factor for atherogenesis. The results also suggest expanding the notion of bacterial ‘privileged niche’ to include vascular tissue, where bacteria can persist for extended periods of time, feeding the chronicity of inflammation.

Polybacterial infection fits well with the proposed aggregate notion of ‘pathogen burden’ [15]. Most of the organisms we cultivated from atheromas (*P. acnes*, *S. epidermidis* and *S. infantis*) can be located to different bodily sites, including periodontium, however the presence of *P. gingivalis* is consistent, specifically with periodontal infection. Overall, our findings support the hypothesis of direct invasion of systemic tissues predominantly with oral cavity pathogens.

These observations can explain a model of atherogenesis whereby inflammation is initiated and/or exacerbated by bacteria that can be disseminated during bacteremia or while internalized in phagocytic cells. The novelty is that a variety of pathogens have now been isolated and identified from atheromatous tissues and in the proposed actual mechanism of bacterial persistence in such tissues. Notably, the bacteria can persist intracellularly for an extended period of time in uncultivable stage of dormancy. However, these dormant organisms can become activated, especially in phagocytic cells, switching into a metabolically active state, allowing them to escape the host to infect new host cells. These are the phenomena that underlie the bacterial persistence in vascular tissue, coaxing host defense into an injurious response and ultimately contributing to the chronicity of the vascular inflammations.

Our model further suggests that the failure of antibiotics to reduce ischemic events may be due to dormant unculturable (therefore antibiotictolerant) bacteria that can explain the existing body of epidemiological evidence and that their cultivation and the characterization of their systemic pathogenicity will open novel avenues for diagnosis and treatment of CVD.

Future perspective

It is noteworthy that in other complex conditions the anti-infective approach is now bearing fruit. *H. pylori* has been identified as an underlying agent of duodenal ulcers and antimicrobial therapies are now the standard treatment modality [118]. Notably, that association was discovered before the mechanism of disease was clarified [119]. Similarly, we intend to adopt this attitude to advance the field of vascular medicine. We have many reasons to expect that within 10 years, the identification of the pathogens predominantly associated with vasculopathies, and the introduction of point-of-care diagnostics to determine the risk of development of CVD and the CVD etiology in the individual. Concurrently, adoption of existing antimicrobials for treatment and clinical trials to determine treatment regimens should be initiated based on the individual's specific etiologic agents of disease and their established susceptibility in dormant state to specific drugs.

Box 1. Autophagy

- Autophagy is a trafficking pathway delivering cytoplasmic material to autophagosomes for recycling via fusion with lysosomes and degradation of the contents [120,121]. Recently, autophagy has been recognized as a critically important intracellular surveillance system for recognition and eradication of intracellular pathogens. It is regulated by amino acid concentrations via the mTOR kinase. Autophagy is emerging as a crucial defense mechanism, where intracellular sensors Nod1 and Nod2 are critical for the autophagic response to invasive bacteria [122]. Any bacteria not able to usurp autophagosomes ultimately undergo degradation. For example, Toll-like receptor activation of human macrophages upregulates expression of the vitamin D receptor and the vitamin D1-hydroxylase genes, leading to induction of the antimicrobial peptide cathelicidin and killing of intracellular *Mycobacterium tuberculosis* [123]. Cathelicidin is shown to mediate vitamin D3-induced autophagy [124].

Box 2. Lipid rafts

- Lipid rafts are small (10–200 nM) detergent-insoluble domains in the plasma membrane, enriched in cholesterol, sphingolipids and glycosylphosphatidylinositol anchored proteins. Besides a role in signal transduction, secretion and apical/basolateral sorting in polarized cells, these regions are now thought to play a role in the adhesion and uptake of bacterial pathogens [125].
- Bacterial pathogens can enter host cells via the classical endosome–lysosome route involving clathrin-coated pits or co-opting the endocytic properties of lipid rafts and caveolae (defined as caveolin-enriched lipid rafts). The role of lipid rafts in bacterial pathogenesis is complex. Lipid rafts are shown to be recruited at the *Listeria* entry site since lipid markers (glycosylphosphatidylinositol-linked proteins and modified peptides) are detected at that site [126]. Bacteria entering via lipid rafts may be targeted to different intracellular localizations, thus they do not undergo lysosome fusion, therefore lipid raft-mediated invasion may lead to intracellular survival and bacterial dissemination. Bacterial invasion through lipid rafts can also lead to secretion of inflammatory mediators and to apoptosis [127].

Box 3. Latent state

- Internalization of many types of bacteria can produce a 'privileged niche', where they may not only replicate and disseminate, but also persist in a dormant, nonreplicating state, sheltered from humoral and cellular immune responses, the most studied case being *Mycobacterium tuberculosis* [129].
- Latent state is typical for a chronic bacterial infection and bacteria that are highly prevalent, able to internalize and to persist intracellularly. Overall, the issue of bacterial persistence in inflammatory lesions is poorly understood. Latency as a nonreplicating quiescent phase of bacterial persistence in the metazoan host is recognized as the central problem in controlling pathological conditions including nephritis, rheumatic fever, aphthous stomatitis, idiopathic hematuria, Crohn's disease, Whipple's disease and bacillary angiomatosis [130]. *Legionella pneumophila* also persists intracellularly in a dormant cyst-like transmissible form resistant to antibiotics and detergent [131]. It is estimated that the most studied latent pathogen, *M. tuberculosis*, infects over 2 billion people today [132]. It is encouraging that latency *M. tuberculosis* antigens are targeted by immune system during persistent infection and have also been associated with immunity against latent *M. tuberculosis* infection [133].

Executive summary

Atherosclerosis as an inflammatory disease

- Atherosclerosis (AS) is a chronic inflammatory condition that cannot be fully explained with conventional risk factors.

Vascular infectology: are infections a risk factor for AS?

- Epidemiological and seroepidemiological evidence is available.
- However, there is an insufficient mechanistic link between infections and atherogenesis.

Oral diseases & systemic inflammations

- Epidemiological and seroepidemiological evidence supports peridontal link.
- Periodontal bacterial burden and some specific pathogens have been associated with clinical measures of AS.
- Two parallel avenues exist for metastatic pathogen dissemination, bacteremia and leukocyte-mediated systemic spread.

Response to injury model of AS

- This model describes a vascular inflammation leading to atherogenesis and to thrombosis.
- However, evidence of bacterial presence at the diseased site (Koch's postulate) is required to determine a causal relationship.

Bacterial invasion of vascular tissue

- There are multiple mechanisms by which infectious agents can be implicated in the etiology of various vascular conditions including direct microbial

invasion of endothelial cells, host cell activation and stimulation of leukocyte influx.

- Tissue invasion provides bacteria with: a 'privileged niche' with access to host protein (nutritional) and iron substrates; a sequestration from the humoral and cellular immune response; and a means for persistence that is essential for a chronic pathogen.
- A variety of bacterial species have been shown to invade human endothelial and smooth muscle cells. Extended intracellular survival (persistence) has been observed with some bacterial pathogens, in some cases due to usurping of intracellular trafficking to avoid lysis.
- Bacterial transmission between various vascular cell types has also been demonstrated.

Proatherogenic sequelae of bacterial presence in vascular cells

- Interaction of endothelia with bacteria or their products can lead to endothelial activation/dysfunction and secretion of proinflammatory mediators, the first stage of atherogenesis, followed by leukocyte migration and extravasation.
- Bacteria can trigger apoptosis and necrosis in the host cells, leading to release of matrix metalloproteinases, accelerating the inflammation and causing plaque destabilization.
- Plaque rupture can lead to platelet-aggregating bacteria, tissue factor and other prothrombotic substances gaining access to the circulation and trigger blood coagulation.
- Antibacterials to oral pathogens have been shown to prevent atherogenic process in animal models.
- Animal models have generally supported the *in vitro* investigations; however, they are not always applicable to human disease.

Association of bacteria with atherosclerotic lesions

- More than 50 bacterial 16S rDNA fingerprints have been found in atheromatous tissue.
- However, DNA does not mean disease. To fulfill Koch's postulate, the cultivation of microorganisms from diseased tissue has been demonstrated, providing mechanistic data linking infectious organisms to cardiovascular disease.
- Fewer bacterial species have been cultivated that DNA signatures have been found, thus reducing the number of false positives.

Model of bacterial infection-mediated atherogenesis

- Bacteria invade vascular tissue either directly (bacteremia), or intracellularly, within migrating leukocytes that extravasate at the atheromatous site. Bacteria can persist inside the host cells for an extended period of time, activating the proinflammatory cascade.
- Within 24–72 h the bacteria switch into a dormant uncultivable stage, in order to sustain bacterial numbers and persist.

- Some bacteria escape from the dormant stage, exiting into intracellular space and invading adjacent host cells, becoming transiently invasive and cultivable, inducing and perpetuating persistent low-grade inflammation.
- The return to cultivable state (resuscitation) specifically occurs after engulfment by phagocytes, which additionally may lead to metastatic dissemination, injurious response, apoptosis, necrosis and chronicity of the disease.
- Importantly, dormant bacteria have low-metabolic activity, thus they lack a target for antibiotics and therefore are drug-tolerant.
- Supporting this model, periodontal treatment reduces systemic inflammatory markers, improves lipid profiles, improves endothelial function, decreases C-reactive protein concentrations and decreases intima-media thickness.

Further main unresolved issues

- The mechanism of bacterial intracellular persistence and resuscitation.
- The prevalent in atherosclerotic plaque species (the AS microbiome).
- The genetic determinants specific for bacterial strains adapted for vascular sites.
- The drug susceptibility of intracellular and dormant bacteria.

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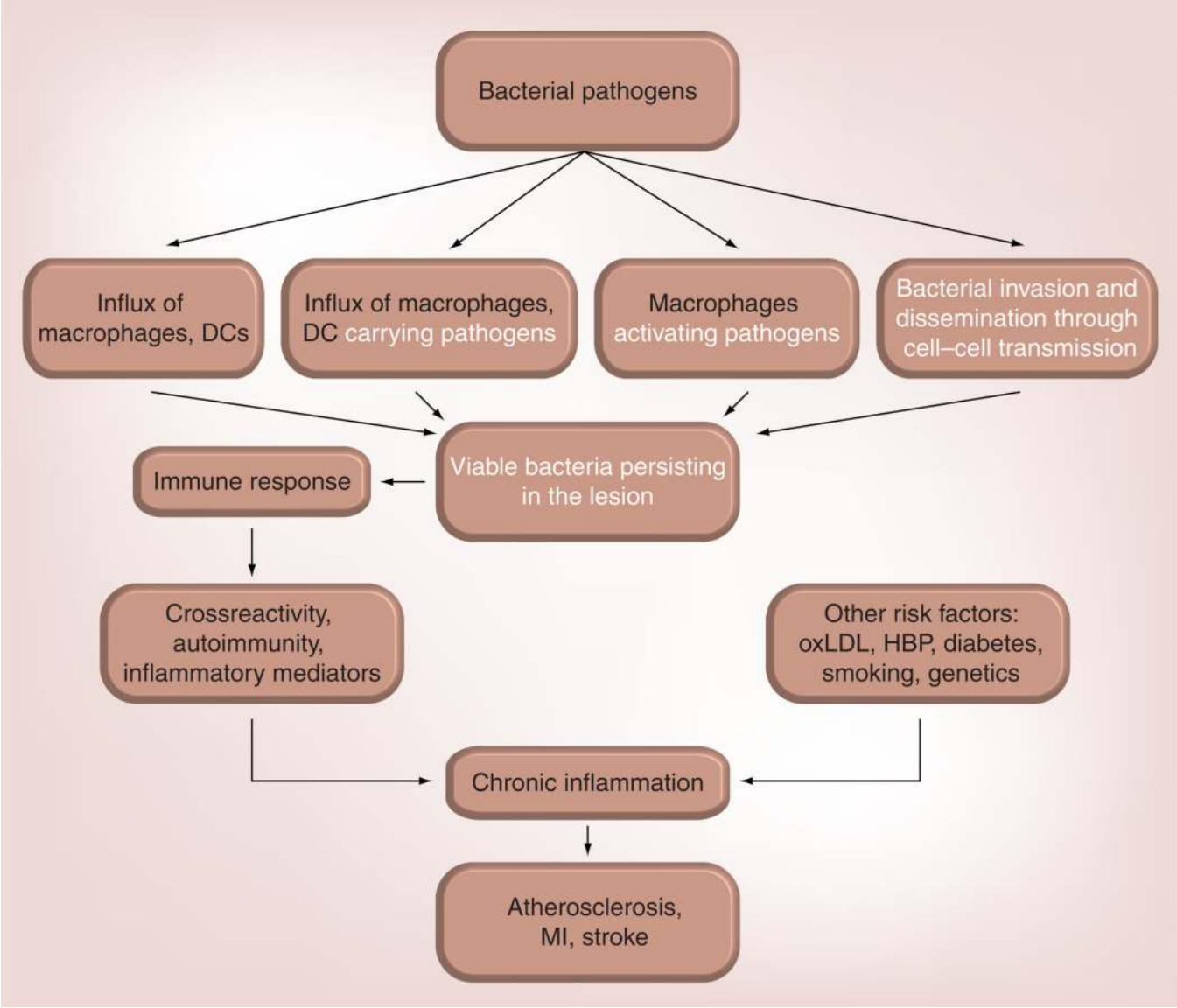

Figure 1. Variety of avenues by which pathogens can contribute to atherogenesis

New insights on the plausible avenues that can be used by bacterial pathogens to initiate and maintain the focal inflammatory lesion that also involve inflammatory mediators and innate and adaptive immunity. The persistence of intracellular bacteria equals constant or repetitive injury that may ultimately lead to necrosis, plaque rupture, myocardial infarction or stroke. In white, contribution of bacteria as suggested by the latest data supporting the model, as discussed in this review.

DC: Dendritic cell; HBP: High blood pressure; MI: Myocardial infarction.

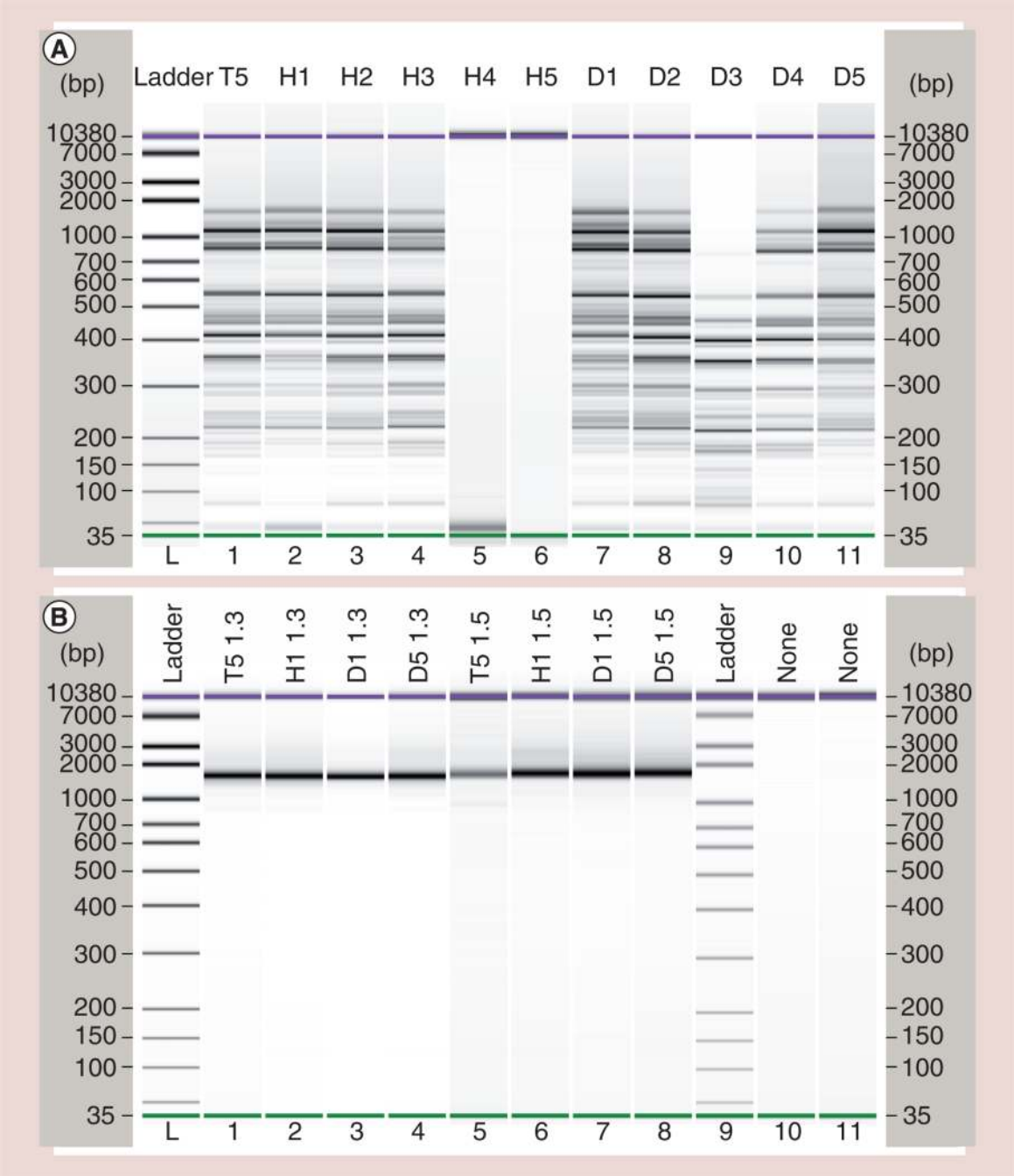

Figure 2. 16S rRNA-specific DNA fragments in atheromata

The results of **(A)** the 16S rDNA PCR and **(B)** the 1.5 kb 16S rDNA-specific gel extracted bands as they appear after the run on the Agilent Bioanalyzer. The fragment analysis shows the absence of 16S-specific bands in the two healthy control tissue samples in the middle (H4 and H5), however, the amplicons that appear in the matching diseased D4 and D5 samples (at right end) are indicative of the presence of bacteria in these samples **(A)**. This suggests an absence of bacterial DNA in healthy arterial tissues, #4 and 5.

Reproduced from [Kalachikov S, Rafferty B, Kozarov E (2012), Manuscript in preparation].

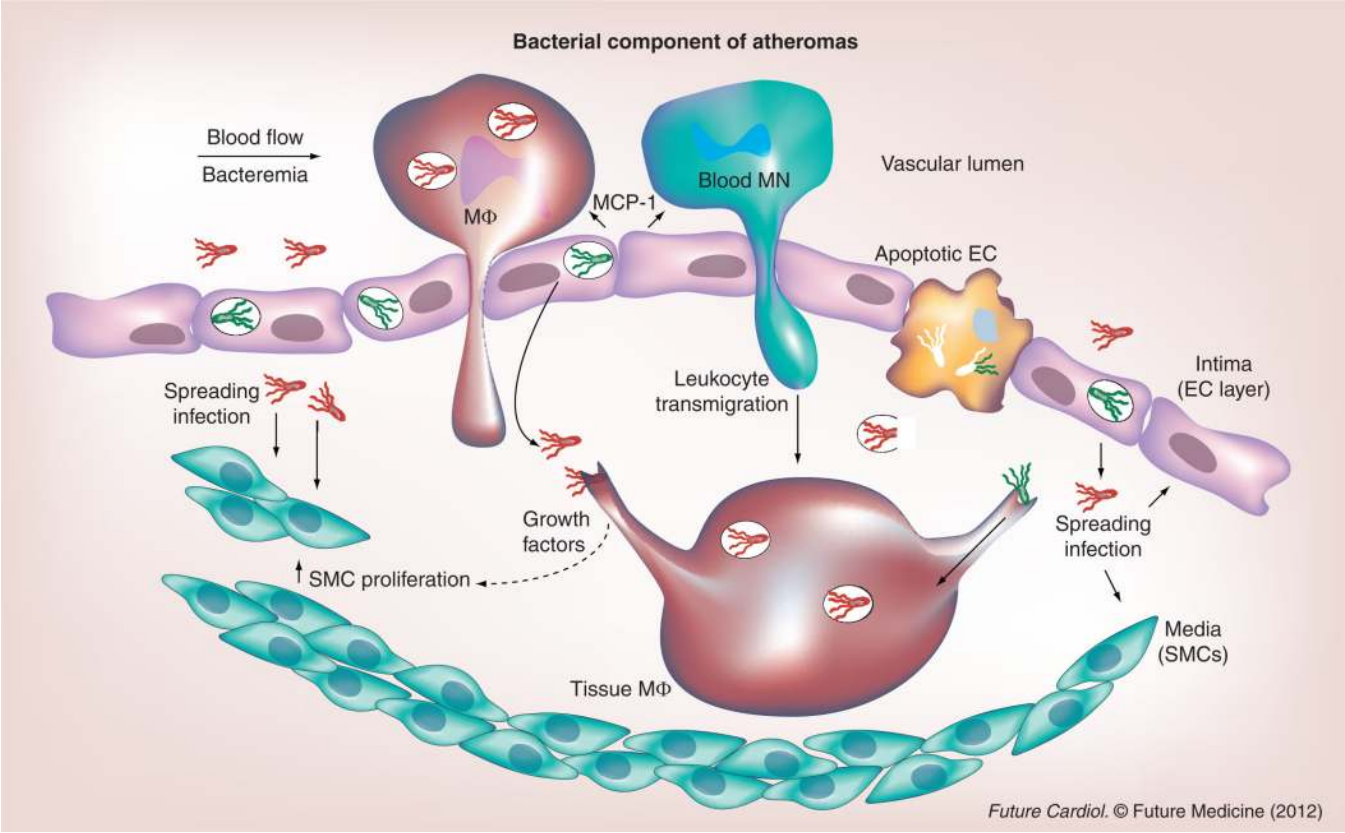

Figure 3. Model of bacterial infection-mediated component of atherogenesis presenting a bacteremic and macrophage-mediated exacerbation of infection

Depicted are the tunica intima, a monolayer of ECs over a basal lamina and the tunica media containing SMCs. Represented on the left is the bacteremic microbial invasion of ECs. Within 24–72 h the invading intracellular bacteria turn into noncultivable state (in green). Endothelial activation is represented as a release in the vascular lumen of proinflammatory mediators such as MCP-1. The mediators activate circulating blood MNs and Mf, promote their local adhesion and diapedesis leading to transmigration into the lesion (in the center). Systemic Mf (on the left) can carry internalized persisting bacteria, thus contributing to the bacterial dissemination. The activation of noncultivable bacteria during vascular cell–cell transmission and the spreading of infection to adjacent ECs and to SMCs is shown on the left and right. Additional bacteria are released in the atherosclerotic core following apoptosis and necrosis of host cells (on the right). The phagocytosis of bacteria by a monocyte maturing into intimal tissue macrophage and the activation of dormant noncultivable bacteria into the active invasive stage (resuscitation, from green to red), following their internalization is shown in the center. Growth factors released from the phagocytes promote SMC proliferation and migration (neointimal formation). For clarity, only our novel paradigm suggesting the spreading, persistence in the dormant state and reactivation of bacteria within phagocytes as a root for chronicity of inflammation is depicted. For previously described mechanisms of atherogenesis such as endothelial activation, surface receptors, blood leukocyte transmigration, lipid uptake, foam cell formation, cell proliferation, vasa vasorum neovascularization, cell death, plaque rupture and blood coagulation/thrombus formation there are excellent visual presentations [128].
EC: Endothelial cell; Mϕ: Macrophage; MN: Monocyte; SMC: Smooth muscle cell.